

Amendments to the Specification:

Please replace the title on page 1 with the following amended title:

C1 -- METHOD OF ISOLATION AND PURIFICATION OF TRYPSIN FROM PRONASE  
PROTEASE AND USE THEREOF --

Please replace paragraph [001] with the following amended paragraph:

C2 [001] The present invention is directed to methods of isolation and purification of *Streptomyces griseus* trypsin (SGT) from ~~pronase~~ PRONASE protease in a single affinity chromatography step and uses of the purified SGT.

Please replace paragraph [004] with the following amended paragraph:

C3 [004] ~~Pronase~~, a PRONASE protease from the microbial organism *Streptomyces griseus* (S.g.), is a commercially available alternative to trypsin prepared from animal tissues. ~~Pronase~~ PRONASE protease has been used for the preparation of primary cell cultures from tissues and for the detachment of cells from surfaces, microcarrier cell cultures and growth of VERO cells in suspension in serum-free media (Weinstein 1966. Exper. Cell Res. 43:234-236; Manousos et al. 1980. In vitro 16:507-515, Litwin 1992. Cytotechn. 10:169-174). The exact mechanism of its action is not known. ~~Pronase~~ PRONASE protease is known to be a mixture of different enzymes, including various types of endopeptidases, (serine and metalloproteases), exopeptidases (carboxypeptidase and aminopeptidase), neutral protease, chymotrypsin, trypsin, carboxypeptidase, aminopeptidase, and neutral and alkaline phosphatase.

Please replace paragraph [005] with the following amended paragraph:

C4 [005] After enzyme treatment, the activity of trypsin is usually neutralized by the addition of fetal calf serum, which contains a number of specific and non-specific protease inhibitors. However, media free of serum and protein (particularly from mammalian sources), are preferred in cell culture media used for production of vaccine and therapeutic proteins. Therefore, use of serum-free media, which are devoid of any trypsin inhibitor activity, makes it necessary to identify new sources of inhibitor activity. Because ~~Pronase~~ PRONASE protease is a mixture of a

variety of proteases, inhibition of protease activity requires a mixture of different inhibitors, leading to a very complex and expensive process. The protein load arising from use of ~~Pr~~onase PRONASE protease and the composition of inhibitors in a serum-free culture therefore would be much higher compared to a culture using mammalian-derived trypsin and specific trypsin-inhibitor. Furthermore, the addition of ~~Pr~~onase PRONASE protease to the culture medium would also adversely effect the purification process, because more protein is present in the medium.

Please replace paragraph [006] with the following amended paragraph:

[006] The trypsin-like activity of ~~Pr~~onase PRONASE protease, commonly known as *Streptomyces griseus* trypsin (SGT) shows a sequence identity of approximately 33% to bovine trypsin (Olafson et al. 1975. Biochem 14:1168-1177). *Streptomyces griseus* trypsin has been purified by chromatographic techniques using different types of ion-exchange resins. These methods typically use stable matrices, which minimise the problem of bleeding of the ligand into the product during elution. These methods, however, have relatively low selectivity, leading to purification factors in the range of <10. As a result, to achieve a high degree of purity, several steps have to be combined, which in turn may cause autodigestion of the trypsin and therefore loss of activity. Purification by ion-exchange chromatography on CM-Sephadex, with further purification performed by rechromatography on a ion exchange column has been described by Jurasek et al. (1971. Can. J. Biochem. 49:1195-1201) and Olafson et al. (1975a. Biochem. 14:1168-1177; 1975b, Biochem. 14: 1161-1167). Miyata et al. (1991. Cell Structure and Function 16:39-43) describe a three step cation exchange chromatography process to purify SGT. SGT is found to migrate as a single band in PAGE with a molecular weight of about 30,000 and having an esterase activity higher than bovine trypsin as determined by BAEE assay. However, even SGT purified by three step chromatography purification methods was found to be slightly contaminated by carboxypeptidase B-like activity.

Please replace paragraph [007] with the following amended paragraph:

[007] SGT has also been purified from ~~Pr~~onase PRONASE protease by affinity chromatography using oligopeptides derived from tryptic digest of salmine as highly specific

C6  
ligand for SGT. Elution of the trypsin-like activity from the mixture of protease in ~~Pr~~onase PRONASE protease with HCl revealed purified SGT which was, however, found to be contaminated by carboxypeptidase B-like activity (Kasei et al. 1975. J. Biochem. 78.:653-662; Yokosawa et al. 1976. J. Biochem. 79:757-763). For analytical purposes only, SGT was also separated from ~~Pr~~onase PRONASE protease by affinophoresis using benzamidine as a ligand (Shimura et al. 1982. J. Biochem.92:1615-1622).

Please replace paragraph [008] with the following amended paragraph:

C7  
[008] There exists a need for a simple large-scale method for isolation and separation of the active trypsin-like fraction of ~~Pr~~onase PRONASE protease. This would allow a controlled system for use in cell culture methods and provide a defined activity of the fraction from a microbial source, which would not bear the risk of contaminants of human pathogens.

C8  
Please replace paragraph [010] with the following amended paragraph:

[010] It is therefore an object of the present invention to provide for a method for isolation of purified Streptomyces griseus trypsin (SGT) from ~~Pr~~onase PRONASE protease.

C9  
Please replace paragraph [017] with the following amended paragraph:

[017] Fig. 2 shows the SDS-PAGE of unpurified Streptomyces griseus ~~Pr~~onase PRONASE protease and purified SGT, with lane 1: unpurified ~~Pr~~onase PRONASE protease, lane 2: flow through of the affinity chromatography and lane 3: purified Streptomyces griseus trypsin after affinity chromatography and elution with 1 M arginine.

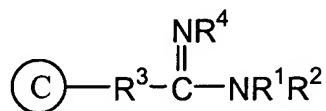
C10  
Please replace paragraph [021] with the following amended paragraph:

[021] In one embodiment, the present invention is directed to a method of isolating SGT by a single chromatography step by contacting the ~~Pr~~onase PRONASE protease to an immobilized affinity moiety (e.g., an amidine, a guanidine, or an amine containing species) and eluting the trypsin selectively from the column with a member of the same classes of compounds used as the affinity moiety. The eluting agent acts as a competitor to the affinity moiety that is immobilized

Q10 to the carrier for the SGT. Thus, in some embodiments, the eluting agent is selected to have greater affinity for the SGT than the affinity moiety.

Please replace paragraph [022] with the following amended paragraph:

[022] According to some embodiments of the invention the ~~Pronase~~ PRONASE protease is contacted with an immobilized affinity column, which includes an amidine. As used herein, the term "amidine" includes amidine and derivatives thereof (*e.g.*, in which the hydrogen atom attached to the amidino nitrogen (=NH) is replaced by substituted or unsubstituted alkyl groups, substituted or unsubstituted heteroalkyl groups, substituted or unsubstituted aryl groups, and substituted or unsubstituted heteroaryl groups). In these embodiments, the amidine has the structure:



in which the circle with the inset "C" represents a component of the column or other solid support. The symbols R<sup>1</sup>, R<sup>2</sup>, and R<sup>4</sup> are each members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, and substituted or unsubstituted heteroaryl groups. R<sup>3</sup> can be either present or absent and can have any of the identities set forth above, with the exception of H. Representative amidine derivatives include substituted or unsubstituted benzamidine species. Amidines that can be used include, but are not limited to, benzamidine hydrochloride; p-aminobenzamidine dihydrochloride; APMSF hydrochloride; 4- amidinophenylmethanesulfonyl -fluoride HCl; bis(5-amidino-2-benzimidazolyl)methane; a,a'-bis (4-amidino-2-iodophenyl)-p-xylene; 1,2-bis(5-amidino-2-benzofuranyl)ethane; and 6-amidino-2-(4-amidinophenyl)benzo-[β]thiophene.

Please replace paragraph [030] with the following amended paragraph:

C12 [030] In the method provided, SGT is selectively purified from a mixture of various proteases, some of them, particularly chymotrypsin, have similar physiochemical characteristics which are difficult to separate with known methods. However, with the method of the invention using

C12  
arginine in a concentration between about 0.5 M and about 1.2 M in the eluant, SGT is selectively separated from other proteases in the ~~Pronase~~ PRONASE protease mixture.

Please replace paragraph [034] with the following amended paragraph:

C13  
[034] In the methods of the invention, the ~~Pronase~~ PRONASE protease is preferably solubilized. The solubilizing agent can be a buffer, wherein the buffer solution can be a Tris-HCl buffer, phosphate buffer, or sulfate buffer. Optionally, the buffer can comprise a salt, such as sodium salts. The buffer preferably has a pH between about 6.0 and about 8.0. The ~~Pronase~~ PRONASE protease is contacted with the carrier matrix and SGT is selectively eluted by competitive elution, typically with arginine.

Please replace paragraph [065] with the following amended paragraph:

C14  
[065] Having now generally described this invention, the invention can be understood by reference to the following examples which are provided herein for purposes of illustration only and are not intended to be limiting.

EXAMPLE 1[[:]]

Purification of Streptomyces griseus trypsin from ~~Pronase~~ PRONASE protease

A. Ion exchange chromatography

Please replace paragraph [066] with the following amended paragraph:

C15  
[066] 30 g of ~~Pronase~~ PRONASE protease (Boehringer Ingelheim) is dissolved in Buffer A (0.02 pyridin, pH 5.0) to a final concentration of 40 mg/ml ~~Pronase~~ PRONASE protease. 25 ml of the solution is subjected to cation exchange chromatography on CM Sepharose Cl 6B (Pharmacia) equilibrated with buffer A). The elution is performed at room temperature using a linear gradient with buffer A (0.02 M pyridin) and buffer B (0.75M pyridin pH 5.0) with 5 times the column volume.

Please replace paragraph [069] with the following amended paragraph:

[069] The chymotrypsin activity is measured by chromogenic assay using 3-carboxymethoxypropionyl-L-arginyl-L-propyl-L-tyrosine-p-notroaniline hydrochloride (S-2586, Chromogenix). The results are expressed  $\Delta$  absorbance units per minute ( $\Delta$  A/min).

TABLE 1

Purification of ~~Pronase~~ PRONASE protease by ion exchange chromatography

| Streptomyces griseus <del>Pronase</del><br><u>PRONASE</u> protease | <del>Pronase</del> - <u>PRONASE</u> protease<br>unpurified | Purified fraction                                |
|--|--|--|
| Protein (g)  | 1  | 0.08   |
| Specific activity U/mg   | $1.6 \times 10^3$  | $16.5 \times 10^3$                               |
| Recovery U in %  | 100  | 70   |
| Stability by SDS-PAGE  | n.d.   | Unstable , low molecular<br>weight fragmentation |
| Inhibition by soy bean inhibitor<br>(% inhibition)                 | n.d.   | $90 \pm 0.1$                                     |
| Chymotrypsin activity<br>( $\Delta$ A / min)                       | 450  | 38   |

\* n.d. not determined

Please replace paragraph [070] with the following amended paragraph:

[070] Table 1 shows that the fractions containing a protein having trypsin-like activity, as determined by inhibition test with soy bean inhibitor, can be purified by ion exchange

chromatography with a specific activity which is about 10 times higher than of ~~Pronase~~ PRONASE protease and with a recovery of about 70%. However, the protein is unstable and shows not a single band, but various bands in SDS-PAGE. This is indicative of fragmentation and autocleavage of the protein (Fig. 1, lane 1).

017  
B. Affinity chromatography on immobilized benzamidine

Please replace paragraph [071] with the following amended paragraph:

018  
[071] A Benzamidine Sepharose 6B fast flow (Pharmacia) column equilibrated with buffer A (50 mM Tris, 0.5 M NaCl pH 7.0) is loaded with 40 ml of a ~~Pronase~~ PRONASE protease solution (75 ~~mg/ml~~ mg/ml, buffer A). Elution is performed with Buffer B (50 mM Tris, 0.5 M NaCl pH 7.0, 10 mM benzamidine hydrochloride pH 7.0), buffer C (0.5 M NaCl, 0.6 M arginine, pH 5.5) or buffer D (0.5 M NaCl, 1 M arginine, pH 5.5).

Please replace paragraph [072] with the following amended paragraph:

019  
The fractions collected are tested for inhibiting properties using soy bean inhibitor, as well as trypsin and chymotrypsin activity as described in Example 1 A. The specific activity is determined as units of enzyme activity per mg protein.

TABLE 2

Purification of ~~Pronase~~ PRONASE protease by affinity chromatography on immobilized benzamidine and elution with benzamidine

| Affinity chromatography and elution with benzamidine (Buffer B)    |   |                   |
|--|---|-------------------|
| Streptomyces griseus <del>pronase</del><br><u>PRONASE protease</u> | <del>Pronase-PRONASE protease</del><br>unpurified | Purified fraction |
| Protein (g)  | 3   | 0.13              |

Q19

|  |                   |                                 |
|--|-------------------|---------------------------------|
| Specific activity U/mg                               | $1.6 \times 10^3$ | $19 \times 10^3$                |
| Recovery U in %                                      | 100               | 60                              |
| Stability by SDS-PAGE                                | stable            | <del>Stable</del> <u>stable</u> |
| Inhibition by soy bean inhibitor<br>(% inhibition)   | n.d.              | $99.98 \pm 0.1\%$               |
| Chymotrypsin activity<br>( $\Delta A / \text{min}$ ) | n.d.              | 0.1                             |

Please replace paragraph [073] with the following amended paragraph:

The results summarized in Table 2 show that by competitive elution with benzamidine, 60% of purified trypsin-like activity of ~~Pronase~~ PRONASE protease is recovered with a specific activity of about  $140 \text{ U}/\mu\text{g}$  U/ $\mu\text{g}$  protein. However, the purified trypsin-like protease containing fraction is preferably further purified and the benzaminide removed prior to use in processes which involve cell culture growth or production of biologicals for application in humans.

TABLE 3

Purification of ~~Pronase~~ PRONASE protease by affinity chromatography on immobilized benzamidine and elution with 0.6 M arginine and 1M arginine

Q20

| Affinity chromatography and elution with 0.6 M arginine (Buffer C) |  |                   |
|--|--|-------------------|
| Streptomyces griseus <del>Pronase</del><br><u>PRONASE</u> protease | <del>Pronase</del> - <u>PRONASE</u> protease<br>unpurified | Purified fraction |
| Protein (g)  | 3  | 0.13              |
| Specific activity U/mg   | $1.6 \times 10^3$  | $26 \times 10^3$  |
| Recovery U in %  | n.d.   | 63                |



|  |  |                        |
|--|--|------------------------|
| Stability by SDS-PAGE  | stable   | <u>Stable stable</u>   |
| Inhibition by soy bean inhibitor<br>(% inhibition)                 | n.d.   | 99.89 ± 0.1%           |
| Chymotrypsin activity<br>(Δ A / min)                               | n.d.   | <0.1                   |
| Affinity chromatography and elution with 1 M arginine (Buffer D)   |  |                        |
| Streptomyces griseus <del>Pronase</del><br><u>PRONASE protease</u> | <del>Pronase</del> <u>PRONASE protease</u><br>unpurified | Purified fraction      |
| Protein (g)  | 3  | 0.13                   |
| Specific activity U/mg   | 1.6 x 10 <sup>3</sup>                                    | 46.5 x 10 <sup>3</sup> |
| Recovery U in %  | n.d.   | 71%                    |
| Stability by SDS-PAGE  | stable   | <u>Stable stable</u>   |
| Inhibition by soy bean inhibitor<br>(% inhibition)                 | n.d.   | 99.99 ± 0.1%           |
| Chymotrypsin activity<br>(Δ A / min)                               | n.d.   | <0.1                   |
| LAL (EU / 1000U)   | 88   | < 4                    |

Please replace paragraph [074] with the following amended paragraph:

[074] As can be seen from results in Table 3, about 63% of the initial trypsin-like activity of ~~Pronase~~ PRONASE protease is recovered when using a buffer comprising 0.6 M arginine, whereas about 71% is recovered with a buffer comprising 1M arginine. The purified SGT eluted with arginine from a benzamidine affinity carrier also has a higher specific activity compared to SGT obtained by ion exchange chromatography or elution with benzamidine from a benzamidine

(121) carrier. Further, a product of higher purity and specific activity is obtained when a buffer comprising increasing molarity of arginine is used.

Please replace paragraph [075] with the following amended paragraph:

(122) [075] Samples of the unpurified *S. griseus* ~~Pronase~~ PRONASE protease, the flow through of the affinity chromatography column and the purified SGT of the elution with 1 M arginine are analysed by SDS-PAGE (Fig. 2).

Please replace paragraph [079] with the following amended paragraph:

(123) [079] The protein content and the esterase activity of each enzyme is measured by the use of BAEE (N-benzoyl-L-arginine ethyl ester) as a substrate of porcine trypsin (purity grade, type IX, crystallized, Sigma), ~~Pronase~~ PRONASE protease (purity grade, Boehringer Ingelheim) and purified SGT obtained by affinity chromatography on benzamidine Sepharose 6 B and elution with 0.6 M arginine according to Example 1 B. The results of this and further experiments are summarized in Table 4.

TABLE 4

Specific activity of proteases used for Vero culture experiments

| Protease  | BAEE activity<br>(Units / ml) | Protein<br>( $\mu$ g/ml) | BAEE activity / Protein<br>( $\frac{U}{\mu g}$ ) ( $\frac{U}{\mu g}$ ) |
|---|-------------------------------|--------------------------|--|
| Porcine Trypsin                                     | 12,555                        | 940                      | 13.4   |
| Crude <del>Pronase</del><br><u>PRONASE</u> Protease | 1,844                         | 740                      | 1.5  |
| Purified SGT  | 8,564                         | 330                      | 26.0   |

EXAMPLE 3

C23 Determination of total protein load for subculture of serum and protein-free VERO cells

Please replace paragraph [081] with the following amended paragraph:

C24 [081] Different protein concentrations between 1 µg and 10,000 µg of porcine trypsin (purity grade, type IX, crystallized, Sigma), ~~Prø~~ PRONASE protease (purity grade, Boehringer Ingelheim) and purified SGT having a specific activity as determined in Example 2 are added to static cultures of T-flasks, cells in roller bottles or cells bound to microcarriers. The total protein amount of trypsin, ~~prø~~ PRONASE protease and purified SGT needed for complete cell detachment and subsequent attachment (subculturing) cultures is given in Table 5.

Please replace paragraph [082] with the following amended paragraph:

C25 [082] Table 5 shows that when using purified SGT, the total protein load of protease for cell detachment and passaging in a static culture is reduced to 4%, in roller bottles to 17% and in the microcarrier culture system to 20% compared to the amount needed when using trypsin.

TABLE 5

Comparison of total protein amount of protease needed for complete cell detachment and subculture of VERO cells

| <del>Cell culture type</del> <u>Culture Type</u>                   | <del>Porcine Trypsin</del> <u>Porcine Trypsin</u><br>(µg) | Purified SGT<br>(µg) | Reduction of <del>protein load</del> <u>Protein Load</u> |
|--|---|----------------------|--|
| Static <del>culture</del> <u>Culture</u><br>(150 cm <sup>2</sup> ) | 100   | 4                    | 25 x   |
| Roller Bottle<br>(850 cm <sup>2</sup> )                            | 300   | 51                   | 6 x  |
| Microcarrier Culture<br>(1 liter)                                  | 5,000   | 1,000                | 5 x  |

EXAMPLE 4

Comparison of mammalian-derived trypsin, ~~Pronase~~ PRONASE protease and purified SGT on cell growth

Please replace paragraph [084] with the following amended paragraph:

[084] VERO cells are either grown in T-flasks or on microcarriers (37°C, CO<sub>2</sub> concentration 5-10%). Subculture is performed as determined in Example 3 by using porcine trypsin (purity grade, type IX, crystallized, Sigma) and ~~Pronase~~ PRONASE protease (purity grade, Boehringer Ingelheim) in T-flasks in a final amount of 100µg and in microcarrier culture of 5000 µg. Purified SGT with a specific activity of  $2.6 \times 10^4$  U/mg U/mg is added to T-flask culture in final amount of 4 µg and in microcarrier culture of 1000 µg. Cell attachment and cell growth are measured by visual inspection and counting of non-attached cells and are expressed as proliferation activity. Table 6 shows proliferation activity expressed in % of total amount of VERO cells either grown in T-flasks or on microcarriers.

TABLE 6

Effect of Trypsin, ~~Pronase~~ PRONASE protease and SGT on proliferation activity of cells

| Cell culture type <u>Culture</u><br><u>Type</u> | <u>Protase Protease</u> (µg) |   |                      |
|---|------------------------------|---|----------------------|
| T-flask   | Trypsin<br>100 µg            | <del>Pronase</del><br><u>PRONASE</u><br><u>protease</u><br>100 µg | Purified SGT<br>4 µg |
| Attachment/Growth                               | 90-100 %                     | 90-95 %   | 90-100%              |

C26

|                      |                    |  |                         |
|----------------------|--------------------|--|-------------------------|
| Microcarrier Culture | Trypsin<br>5000 µg | <del>Pronase</del><br><u>PRONASE</u><br><u>protease</u><br>5000 µg | Purified SGT<br>1000 µg |
| Attachment Growth    | 95-100 %           | 70%  | 95-100%                 |

Please replace paragraph [085] with the following amended paragraph:

[085] As shown in Table 6, crude ~~Pronase~~ PRONASE protease did not allow sufficient repeated transfer of Vero cells grown in serum-free medium, whereas purified SGT is as effective as porcine trypsin, but with a significantly reduced final protein load.

C27  
EXAMPLE 5

Virus antigen Production in Vero Cells using purified SGT

5.1 In vivo activation of Influenza virus and virus production in roller bottles